Quantitative Determination of Antibacterial Susceptibility Using BD Phoenix for the Clinical Isolates from IBD Patients and Inhibition Against Biological Chitosan using Resazurin Assay

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Abstract **- Chitosan is a bio polymer which has multiple pharmacological units. Chitosan can also be used to treat various types of infections and diseases. One amongst is ulcerative colitis. The biological chitosan was synthesised using mushroom and the soil isolate Bacillus sp. The production media was prepared and the chitosan was synthesized. The clinical isolates were isolated from IBD stool samples such as E.coli, K.pnuemoniae, P.aeruginosa and S.aureus. Quantitative determination of antibacterial susceptibility using BD Phoenix for the clinical isolates from left – over stool samples of IBD Patients. Results showed that 90 % of the drugs were sensitive to most of the isolates among the analysed 40 drugs. The resistant drug was nalidixic acid to E. coli whereas ampicillin was resistant to K.pneumoniae. Gentamicin and tigecycline were resistant to P. aeruginosa and rifampicin and trimethoprim were found resistant to S. aureus. The antibacterial activity of biological chitosan against E.coli, K.pnuemoniae, P.aeruginosa and S.aureus was evaluated by minimum inhibitory concentration (MIC). A visual reading of the direct REMA, which was the best method for distinguishing resistant and susceptible strains, indicated that the MIC cut-off points for the biological chitosan was 3200 µg/ml.**

Index Terms **- Biological Chitosan, Resazurin and MIC.**

1.INTRODUCTION

Chitin is a major component present mainly in the exoskeleton of crustaceans. Chitosan is recently used in many areas such as pharmaceuticals, drug carrier, food additives and agriculture. It is also used in shampoo, toothpaste and cosmetics [1]. Due to seasonal conditions the variability of chitin to chitosan cost little higher in associated with chemical conversion [2]. Chitosan is widely recognized for its potent antimicrobial activity with, broad spectrum, and high killing rate but low toxicity toward mammalian cells [3]. Although the mode of antimicrobial action of chitosan is not completely understood, it is well established that the molecular structure of chitosan is prerequisite for its antimicrobial activity [4,5]. The antibacterial activity of chitosan is influenced by a number of factors that include the type of chitosan, the degree of chitosan polymerization and some of its other physicochemical properties [6].

The minimal inhibitory concentration (MIC), which is a key indicator of an antimicrobial agent's potency, is defined as the concentration (mgl^{-1}) at which visible growth of bacteria is prevented under defined growth conditions [7]. Well- and disc-diffusion methods have frequently been reported as qualitative indicators for testing the antimicrobial activity of natural products [8]. Such testing methods are standardised by the Clinical and Laboratory Standards Institute (CLSI) for antibiotic testing (The Clinical and Laboratory Standards Institute M100-S22).

2. MATERIALS AND METHODS

A) QUANTITATIVE DETERMINATION OF ANTIBACTERIAL SUSCEPTIBILITY

The BD Phoenix™ Automated Microbiology System is intended for the in vitro rapid identification (ID) and quantitative determination of antimicrobial

susceptibility by minimal inhibitory concentration (MIC). 40 drugs namely Ampicillin, Amoxicillin, Clavulanic Acid, Piperacillin, Tazobactam, Cefuroxime, Cefuroxime Axetil, Ceftriaxone, Cefoperazone, Sulbactam, Cefepime, Ertapenem, Imipenem, Meropenem, Amikacin, Gentamicin, Nalidixic Acid, Ciprofloxacin, Tigecycline, Nitrofurantoin, Colistin, Trimethoprim, Sulfamethoxazole, Aztreonam, Doripenem, Minocycline, Levofloxacin, Cefoxitin Screen, Benzylpenicillin, Oxacillin, Gentamicin High Level (synergy), Inducible Clindamycin Resistance, Erythromycin, Clindamycin, Linezolid, Daptomycin, Teicoplanin, Vancomycin, Nitrofurantoin and Rifampicin have been used for evaluation.

B) ANTIBACTERIAL ACTIVITY OF BIOLOGICAL CHITOSAN USING RESAZURIN ASSAY

A sterile 96 well plate was labelled as 1,2,3,4 and 5. 100ul of sterile Muller – Hinton broth was added in all the wells. The two-fold dilutions of biologically synthesised chitosan were made as 200, 400, 800, 1600 and 3200 µg/ml respectively using sterile distilled water. The plate was wrapped in aluminium foil and incubated at 37 C for 24–48hours. 30µl (100 µg/ml) of resazurin indicator solution was added to each well. Finally, 10µl of each bacterial suspension (5×10^6cfu/ml) was added to each well to achieve a concentration of 5×10^5cfu/ml. The plate was wrapped again in aluminium foil and incubated overnight. The blanks were prepared as follows, Blank 1- The sterility control was maintained with the test compound (chitosan), broth, sterile distilled water and indicator. Blank 2 - Control without drug (bacteria, broth and indicator) was used. Blank 3 -Positive control (ciprofloxacin (10ul) in serial dilution + broth + indicator + bacteria). The colour change from blue to pink or colourless is the upper limit for MIC. (The lowest concentration of the drug prevents the colour change). The highest concentration which shows a colour change from blue to pink is the lower limit.

3. RESULTS AND DISCUSSION

90 % of the drugs were sensitive to most of the isolates among the analysed 40 drugs. The resistant drug was nalidixic acid to E. coli whereas ampicillin was resistant to K.pneumoniae. Gentamicin and tigecycline were resistant to P. aeruginosa and rifampicin and trimethoprim were found resistant to S. aureus (Table 1 A, B, C and D).

TABLE NO. 1: MIC

A. ESCHERICHIA COLI

Patient Name: 13, 13 Patient ID: 04019679
Isolate: 04019679-13 (Approved)

Card Type: AST-N280 Bar Code: 7001203403335584 Testing Instrument: 000014EEE404 (9336)
Setup Technologist: Laboratory Administrator(Labadmin)

Organism Quantity: **Selected Organism: Escherichia coli**

Comments:	

Identification Information			
Organism Origin	Technologist		
Selected Organism	Escherichia coli		
	Entered: Dec 29, 2019 12:21 CST	**By:**	Labadmin
Analysis Messages:			

Susceptibility Information	Card:	AST-N280	Lot Number:	7001203403	Expires:	Mar 13, 2021 12:00 CST
	Completed:	Dec 29, 2019 21:48 CST	Status:	Final	Analysis Time:	9.47 hours

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ampicillin	4	S	Meropenem	<= 0.25	S
Amoxicillin/Clavulanic Acid	<= 2	S	Amikacin	<= 2	S
Piperacillin/Tazobactam	<= 4	S	Gentamicin	<= 1	S
Cefuroxime	4	S	Nalidixic Acid	>= 32	R
Cefuroxime Axetil	4	S	Ciprofloxacin	0.5	I
Ceftriaxone	<= 1	S	Tigecycline	<= 0.5	S
Cefoperazone/Sulbactam	<= 8	S	Nitrofurantoin	<= 16	S
Cefepime	<= 1	S	Colistin	<= 0.5	S
Ertapenem	<= 0.5	S	Trimethoprim/Sulfamethoxazole	<= 20	S
Imipenem	<= 0.25	S			

+= Deduced drug *= AES modified **= User modified

AES Findings:	Last Modified: Apr 12, 2019 19:37 CDT	Parameter Set: Copy of Global CLSI-based+Natural Resistance(2019)
Confidence Level:	Consistent	

B: KLEBSIELLA PNEUMONIAE

Patient Name: 3, 3 Patient ID: 04019746
Isolate: 04019746-3 (Approved)

Card Type: AST-N280 Bar Code: 7001236203649031 Testing Instrument: 000014EEE404 (9336)
Setup Technologist: Laboratory Administrator(Labadmin)

Organism Quantity: **Selected Organism: Klebsiella pneumoniae**

Comments:	

Identification Information			
Organism Origin	Technologist		
Selected Organism	Klebsiella pneumoniae		
	Entered: Dec 31, 2019 10:30 CST	By:	Labadmin
Analysis Messages:			

Susceptibility Information	Card:	AST-N280	Lot Number:	7001236203	Expires:	Apr 15, 2021 13:00 CDT
	Completed:	Dec 31, 2019 18:14 CST	Status:	Final	Analysis Time:	9.48 hours

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ampicillin	16	*R	Meropenem	<= 0.25	S
Amoxicillin/Clavulanic Acid	<= 2	S	Amikacin	<= 2	S
Piperacillin/Tazobactam	<= 4	S	Gentamicin	<= 1	S
Cefuroxime	<= 1	S	Nalidixic Acid	<= 2	S
Cefuroxime Axetil	<= 1	S	Ciprofloxacin	<= 0.25	S
Ceftriaxone	<= 1	S	Tigecycline	<= 0.5	S
Cefoperazone/Sulbactam	<= 8	S	Nitrofurantoin	64	I
Cefepime	<= 1	S	Colistin	<= 0.5	S
Ertapenem	<= 0.5	S	Trimethoprim/Sulfamethoxazole	<= 20	S
Imipenem	<= 0.25	S			

+= Deduced drug *= AES modified **= User modified

AES Findings:	Last Modified: Apr 12, 2019 19:37 CDT	Parameter Set: Copy of Global CLSI-based+Natural Resistance(2019)
Confidence Level:	Consistent	

C: PSEUDOMONAS AERUGINOSA

Patient Name: 13, 13 Patient ID: 01074856
Isolate: 01074856-13 (Approved)

Card Type: AST-N281 Bar Code: 7011106103124929 Testing Instrument: 000014EEE404 (9336)
Setup Technologist: Laboratory Supervisor(LabSuper)

Organism Quantity: **Selected Organism: Pseudomonas aeruginosa**

Comments:	

Identification Information				
Organism Origin	Technologist			
Selected Organism	Pseudomonas aeruginosa			
	Entered: Dec 23, 2019 12:32 CST	By:	LabSuper	

Analysis Messages:
The following antibiotic(s) are suppressed from analysis:
Aztreonam,

Susceptibility Information	Card:	AST-N281	Lot Number:	7011106103	Expires:	Dec 6, 2020 12:00 CST
	Completed:	Dec 24, 2019 03:48 CST	Status:	Final	Analysis Time:	16.07 hours

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ticarcillin/Clavulanic Acid	32	I	Amikacin	<= 2	S
Piperacillin/Tazobactam	<= 4	S	Gentamicin	>= 16	R
Ceftazidime	4	S	Ciprofloxacin	0.5	S
Cefoperazone/Sulbactam	16	S	Levofloxacin	2	I
Cefepime	2	S	Minocycline		
Aztreonam			Tigecycline	<= 0.5	*R
Doripenem	<= 0.12	S	Colistin	<= 0.5	S
Imipenem	0.5	S	Trimethoprim/Sulfamethoxazole		
Meropenem	<= 0.25	S			

+= Deduced drug *= AES modified **= User modified

AES Findings:	Last Modified: Apr 12, 2019 19:37 CDT	Parameter Set: Copy of Global CLSI-based+Natural Resistance(2019)
Confidence Level:	Consistent	

D: STAPHYLOCOCCUS AUREUS

Patient Name: 3, 3 Patient ID: 04019602
Isolate: 04019602-3 (Approved)

Card Type: AST-P628 Bar Code: 5381167403212772 Testing Instrument: 000014EEE404 (9336)
Setup Technologist: Laboratory Supervisor(LabSuper)

Organism Quantity: **Selected Organism: Staphylococcus aureus**

Comments:	

Identification Information				
Organism Origin	Technologist			
Selected Organism	Staphylococcus aureus			
	Entered: Dec 28, 2019 15:02 CST	By:	LabSuper	

Analysis Messages:
The following antibiotic(s) are not claimed:
Gentamicin High Level (synergy),

Susceptibility Information	Card:	AST-P628	Lot Number:	5381167403	Expires:	Feb 5, 2021 12:00 CST
	Completed:	Dec 28, 2019 22:09 CST	Status:	Final	Analysis Time:	13.10 hours

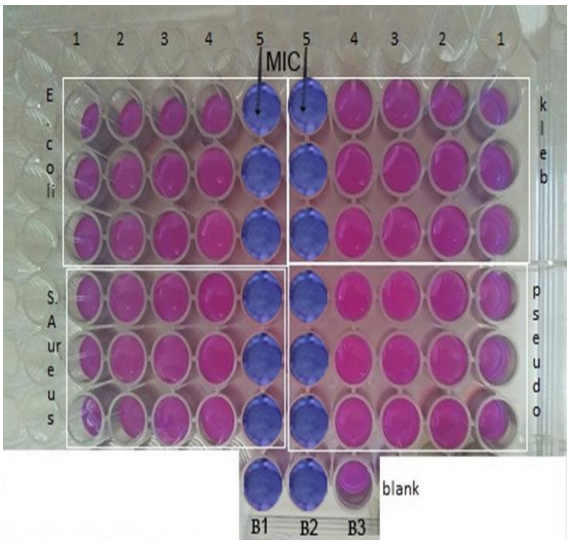
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Cefoxitin Screen	NEG	-	Linezolid	2	S
Benzylpenicillin	0.06	S	Daptomycin	0.5	S
Oxacillin	0.5	S	Teicoplanin	<= 0.5	S
Gentamicin High Level (synergy)			Vancomycin	1	S
Gentamicin	<= 0.5	S	Tetracycline	<= 1	S
Ciprofloxacin	<= 0.5	S	Tigecycline	<= 0.12	S
Levofloxacin	0.25	S	Nitrofurantoin	<= 16	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin	<= 0.03	S
Erythromycin	<= 0.25	S	Trimethoprim/Sulfamethoxazole	>= 320	R
Clindamycin	0.25	S			

+= Deduced drug *= AES modified **= User modified

Minimum Inhibitory Concentration for Standard and synthesised Chitosan by Resazurin Microplate Assay

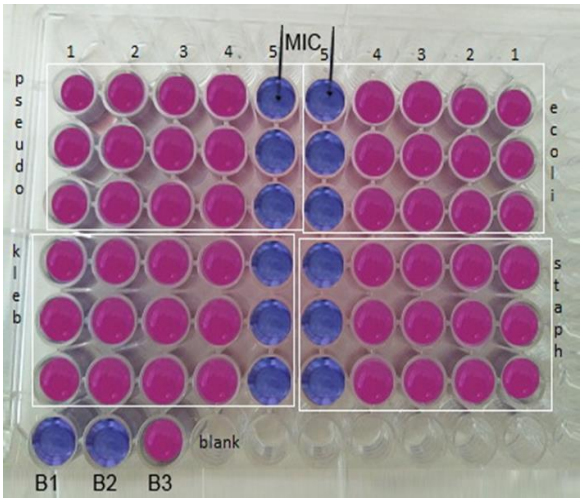
The Minimum Inhibitory Concentration (MIC) of the standard and the synthesized chitosan was compared using the REMA method. Both the samples inhibited the change of colour in the highest concentration out of the tested five concentrations (200, 400, 800, 1600 and 3200 µg/ml respectively). A visual reading of the direct REMA, which was the best method for distinguishing resistant and susceptible strains, indicated that the MIC cut-off points for the biological chitosan was 3200 µg/ml (Figure no. 1 and 2).

FIGURE NO. 1: MIC OF STANDARD CHITOSAN



Note: 1-200, 2-400, 3-800, 4- 1600 and 5- 3200 (µg/ml)

FIGURE NO. 2: MIC OF BIOLOGICAL CHITOSAN



Note: 1-200, 2-400, 3-800, 4- 1600 and 5- 3200 (µg/ml)

In the present study, biological chitosan was synthesised and optimised. MIC plays an important role during the process of screening, prioritizing, and optimizing a chemical series during early drug discovery. Minimum Inhibitory Concentration determination by using Resazurin Microtiter Plate Assay (REMA) method. I have used the Resazurin Microtiter Plate Assay (REMA) method to screen test substances for antimycobacterial activity against E.coli, S.aureus, P.aeruginosa and K. pneumoniae. Resazurin, an oxidation-reduction indicator, has been used to assess viability and bacterial contamination and to test for antimicrobial activity. Results obtained using the REMA assay is faster and less expensive. Bearing in mind considerations of rapidity, low technology requirements and low cost, microplate assays that use Resazurin type compounds have the

potential of becoming the methods of choice for drug susceptibility testing[9].

The MIC of both standard and biological chitosan was found to be 3200 µg/ml. Similar report was also obtained on chitosan nanoparticles against Mycobacterium tuberculosis strains H37Rv was 1200 µg/ml using REMA method[10]. The MIC values ranged from 200 $\mu g \cdot ml^{-1}$for E. Coli to 500 $\mu g \cdot ml^{-1}$ of L. monocytogenes[11]. The drug exhibited antibacterial activity at higher concentrations and MIC of them against E.coli and S.aureus was 177 and 277 µg/ml, respectively[12] which are lined with the existing study. Some researcher has also shown that chitosan generally showed stronger effects for g-positive bacteria (e.g. Listeria monocytogenes, Bacillus megaterium, B. cereus, Staphylococcus aureus, Lactobacillus plantarum, L. brevis, L. bulgaris, etc.) and for g-negative bacteria (e.g. E. coli, Pseudomonas fluorescens, Salmonella typhymurium, Vibrio parahaemolyticus, etc) [13&14]. The other mechanism involves the chelating agent properties of chitin and chitosan and their influence on organism growth. Together with low MW, also high-level of degree of deacetylation enhance the antibacterial activity of chitosan with an improvement of permeabilizing effect and a better electrostatic binding to the bacteria membrane [15].

4.CONCLUSION

In clinical microbiology laboratories, for the detection of clinical isolates such as E.coli, S.aureus, P.aeruginosa and K.pneumoniae from the left over stool samples of IBD patients were correctly interpreted by BD Phoenix TM system and also showed reliable results. The BD phoenix system is an extra ordinary diagnostic tool to determine the quantitative AST of clinical isolates. A new reliable method has been developed for standard chitosan and biological chitosan was determined with a high level of accuracy by using MIC. Chitosan has exhibited a highly amorphous function and has promising antimicrobial susceptibility through preliminary in vitro techniques.

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